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Some isolates of the nematophagous fungus *Pochonia chlamydosporia* promote root growth and reduce flowering time of tomato

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Abstract

The fungal parasite of nematode eggs *Pochonia chlamydosporia* is also a root endophyte known to promote growth of some plants. In this study, we analyzed the effect of nine *P. chlamydosporia* isolates from worldwide origin on tomato growth. Experiments were performed at different scales (Petri dish, growth chamber and greenhouse conditions) and developmental stages (seedlings, plantlets and plants). Seven *P. chlamydosporia* isolates significantly ($P<0.05$) increased the number of secondary roots and six of those increased total weight of tomato seedlings. Six *P. chlamydosporia* isolates also increased root weight of tomato plantlets. Root colonization varied between different isolates of this fungus. Again *P. chlamydosporia* significantly increased root growth of tomato plants under

greenhouse conditions and reduced flowering and fruiting times (up to 5 and 12 days, respectively) *vs* uninoculated tomato plants. *P. chlamydosporia* increased mature fruit weight in tomato plants. The basis of the mechanisms for growth, flowering and yield promotion in tomato by the fungus are unknown. However, we found that *P. chlamydosporia* can produce Indole-3-acetic acid and solubilize mineral phosphate. These results suggest that plant hormones or nutrient ability could play an important role. Our results put forward the agronomic importance of *P. chlamydosporia* as biocontrol agent of plant parasitic nematodes with tomato growth promoting capabilities.

Introduction

Endophytes are generally referred to as mostly facultative plant mutualists, capable to colonize their hosts without causing visible harm symptoms (Schulz *et al.,* 1998; Faeth, 2002). Fungal endophytes may have the ability to improve seed germination, root formation and plant growth via production of phytohormones such as Indole-3-acetic acid (IAA) (Dudeja *et al.,* 2012; Chutima & Lumyong, 2012). Endophytes may also help plants by solubilizing insoluble or sparingly soluble minerals (Altomare *et al.,* 1999). They could also increase plant tolerance to biotic and abiotic stresses (Rodriguez *et al.,* 2004), providing protection against herbivore pests (Crawford *et al.,* 2010) and plant parasitic nematodes (Kerry, 2000). All these properties are of great importance in agriculture (Maciá-Vicente *et al.,* 2009).

The annual worldwide economic losses due to nematodes exceed US $100 billion, an equivalent to 11% of crop production in almost all cultivated plants (Kerry, 2000; Bird & Kaloshian, 2003; Danchin *et al*., 2013). The use of biocontrol agents such as nematophagous fungi have shown to reduce dependence on the use of chemicals for management of *Meloidogyne javanica* on tomato, *Rotylenchulus reniformis* on cotton and *Heterodera schachtii* on sugar beet crops (Sorribas *et al.,* 2003; Wang *et al.,* 2005; Ayatollahy *et al.,* 2008).

The fungal parasite of nematode eggs, *Pochonia chlamydosporia* (*Verticillium chlamydosporium* (Goddard) Gams & Zare, 2001), has emerged as a potential biocontrol

agent of plant parasitic nematodes and can thus be used as component of integrated pest management strategies (Verdejo-Lucas *et al.,* 2003; Tobin *et al.,* 2008; Muthulakshmi *et al.,* 2012; Chaya & Rao, 2012). *P. chlamydosporia* parasitizes egg masses or nematode females (de Leij *et al.,* 1992, Bourne *et al.,* 1996) in the rhizosphere but it is also a worldwide soil fungus (Domsch *et al.,* 1993; Zare *et al.,* 2001). The diversity of *P. chlamydosporia* isolates has been related to the host nematode and the soil environment from where the fungus was isolated (Morton *et al.,* 2003). This variation is also reflected in the growth rate, capacity to produce conidia and dictyochlamydospores and production of extracellular enzymes (Olivares & López-Llorca, 2002; Esteves *et al.,* 2009). *P. chlamydosporia* colonizes endophytically monocotyledon (Maciá-Vicente *et al.,* 2008; Lopez-Llorca *et al.,* 2010) and dicotyledon plant roots (Bordallo *et al.,* 2002; Manzanilla-Lopez *et al.,* 2011; Escudero & Lopez-Llorca, 2012). This fungus has been shown to improve yields of infested nematode crops such as cotton (Wang *et al.,* 2005), chili (Singh *et al.,* 2011), lemon (Deepa *et al.,* 2011), potato (Muthulakshmi *et al.,* 2012), okra (Chaya & Rao, 2012), eggplant (Khan *et al.,* 2012), lettuce (Dias-arieira *et al.,* 2011) and the ornamental plants tuberose (*Polyanthus tuberosa L.*), hollyhock (*Althea rosea L.*), petunia (*Petunia hybrid Vilm.*) and poppy (*Papaver rhoeas L.*) (Rao *et al.,* 2004; Khan *et al.,* 2005). With these advantages, several studies suggest that *P. chlamydosporia* also promotes the growth of some crops. In the study realized by Monfort *et al.* (2005), *P. chlamydosporia* improved the growth of wheat (increasing shoot weight). In barley inoculated with *P. chlamydosporia* root weight, shoot weight and shoot length were higher than control plants (Maciá-Vicente *et al.,* 2009; Rosso *et al.,* 2014). Furthermore, Siddiqui & Shaukat (2003) reported than *P. chlamydosporia* alone or used in combination with *Pseudomonas aeruginosa* increased height of tomato plants in field experiments. Meanwhile, Escudero & Lopez-Llorca (2012) reported an increase of shoot and root weight of tomato seedlings under growth chamber conditions.

Tomato is an important commodity plant for human nutrition and has become one of the most extensively used vegetable crops in the world (Ichihashi & Sinha, 2014). The efficacy of *P. chlamydosporia* as control agent of several nematodes (*Meloidogyne hapla, M. incognita, M. javanica and Globodera pallida*) in tomato plants has been extensively demonstrated in *in-vitro* assays and under glasshouse conditions (Siddiqui & Akhtar, 2009;

Atkins *et al.,* 2009; Dallemole-Giaretta *et al.,* 2012). On the contrary, the ability of the fungus to promote tomato growth and their effects on development tomato stages as germination and flowering time or yield remains unknown.

The aim of this study was to evaluate the effect of nine *P. chlamydosporia* isolates from worldwide origin on tomato plantlets growth, flowering and fruiting times and tomato yield. For this purpose, we carried out laboratory tests with seedlings and plantlets as well as pot experiments with adult plants under greenhouse conditions. In these experiments, we compared the performance of tomato plants inoculated or not with each of the *P. chlamydosporia* isolates. We followed these plants root colonization by *P. chlamydosporia* using microscopy, culturing and quantitative PCR techniques. We also tested plant growth promotion traits such as phosphate solubilization and IAA production by the different isolates of *P. chlamydosporia.*

Material and methods

Pochonia chlamydosporia (Pc) isolates

Eight worldwide isolates of *P. chlamydosporia* var. *chlamydosporia*: Pc21, Pc64, Pc69, Pc75, Pc123, Pc132, Pc399, Pc4624 and one of *P. chlamydosporia* var. *catenulata* (Pcat) were used in our studies. Isolates Pc64, Pc75 and Pc123 were obtained from the Plant Pathology Laboratory collection (University of Alicante, Spain). The host origin and location of the isolates are shown in Table 1. All isolates were grown on corn meal agar (CMA) plates (Becton Dickinson and Company) at 25°C in the dark for 25 days.

Experiment 1: Effect of *P. chlamydosporia* isolates on the germination and development of tomato seedlings

All experiments were carried out using tomato seed (*Solanum lycopersicum* L. cv. Marglobe). To test the effect of fungus-plant early stages, seed germination assays were carried out on Petri dishes with Gamborg´s agar (Gamborg´s B-5 Basal salt mixture,

SIGMA) and this medium diluted 1/10. Both media were supplemented with 1.5% Bacteriological Agar European Type (Cultimed). Tomato seed were surface disinfected in 1% sodium hypochlorite with 0.05% Tween 20 (SIGMA) for 10 min and then washed three times in sterile distilled water as in Bordallo *et al*. (2002). Tomato seeds were finally plated around of 7 day-old colonies of *P. chlamydosporia* isolates on the media described above. Plates with seed and fungi were then incubated at 25°C in the dark for 3 days and then in a growth chamber for further 5 days under the same temperature and a 16/8 h (light/darkness) photoperiod (130 mol/m^2s of intensity) generated by cool white light fluorescent tubes (Sylvania standard F65W). Germination associated parameters (emergence of roots, hypocotyls and cotyledons) were scored daily during 8 days. Seedlings were removed 8 days after inoculation (dai) from the plates and numbers of secondary roots and total fresh weight per plant were also recorded. Ten tomato seed per plate were placed in each culture medium and plates without fungus were used as controls. Three replicate plates per treatment were set and the experiment was carried out twice. *P. chlamydosporia* tomato roots were stained with 0.01% Calcofluor White (Fluka Analytical) to verify colonization. Microscopic images were recorded with a Leica DFC 480 camera. Roots were viewed under UV light using an Olympus BH2-RFCA microscope.

Experiment 2: Effect of *P. chlamydosporia* isolates on growth of tomato plantlets

Tomato seedlings were placed in 100 mL sterile plastic containers with 70 cm^3 of disinfected sand as substrate and irrigated (Escudero & Lopez-Llorca, 2012). Each seedling was inoculated with four 5 mm diameter plugs from the edge of a 20 day-old colony on CMA of a given *P. chlamydosporia* isolates (Macia-Vicente *et al.,* 2008). Non-colonized CMA plugs were used as controls. Seedlings were placed in a growth chamber under the same conditions described in Experiment 1 and were then irrigated immediately and every 48 h for 21 days with autoclaved 1/10 Gamborg's B5 solution. Maximum root length (MRL), Fresh root weight (FRW), fresh shoot weight (FSW), dry shoot weight (DSW) and maximum shoot length (MSL) per plant were then recorded. The experiment consisted of 10 plantlets per treatment and was carried out in triplicate.

Experiment 3: Effect of *P. chlamydosporia* isolates on growth of tomato plants

Tomato plantlets inoculated as before with *P. chlamydosporia* isolates and uninoculated controls were planted in 4.5 L pots each containing 4 kg of non-sterile sand and 30 g of NPK 12-7-8 fertilizer. Each pot was inoculated with 5000 *P. chlamydosporia* chlamydospores.g^{-1} of substrate. Conidia and chlamydospores were obtained from solid substrate inoculated with the different isolates. However, for those isolates which produced few or no chlamydospores, an equivalent amount of conidia was added. Pots were re-inoculated with the same amount of chlamydospores 1 and 2 months later. Plants were grown under greenhouse conditions (15–30 °C, 40% RH) where they were watered as required. Lots of 30 plants were inoculated with each isolate (Table 1) of *P. chlamydosporia* (treatment) and 30 uninoculated plants were left as controls. Pots with treatments were randomized leaving 0.5 m between plants. Three months afterwards (90 dai), plants were harvested and growth parameters (FSW, MSL, FRW, and MRL) were recorded per plant. Greenhouse experiment was carried out twice.

The flowering time was evaluated as in Subramanian *et al*. (2006) and Salvioli *et al*. (2012). Essentially, plants were scored daily until 50% of them had bore their first flower or fruit and these dates were respectively chosen as flowering and fruiting times. Upon harvesting (150 dai) the growth parameters (SFW and RFW) and diameter of tomato fruits per plant were scored and size classified using the scale described by Jones (1999) with some modifications. Fruit diameter was recorded with a digital caliper (Stainless Hardened) and values grouped into five categories: stage 1 (<15 mm), stage 2 (15–30 mm), stage 3 (31–45 mm) stage 4 (>45 mm) and marketable fruit (mature fruit). Flowering experiment consisted of 10 plants per treatment and was carried out in triplicate.

Evaluation of tomato root colonization by *P. chlamydosporia*

Total and endophytic root colonization of *P. chlamydosporia* isolates were measured using culturing techniques as in Maciá-Vicente *et al.* (2008). Six root system from experiment 2 (21 dai) and further six from experiment 3 (90 dai) were used to evaluate endophytic and total colonization (3 per each). To detect endophytic colonization, three roots from experiments 2 and 3 were surface disinfected with 1% sodium hypochlorite for 1 min and washed three times in sterile distilled water and then blotted onto sterile paper. Roots were then cut into 1 cm fragments (10 fragments/plate) and plated

on growth restricting medium (CMA 17 $g.L^{-1}$, NaCl 17.5 $g.L^{-1}$, Rose Bengal 75 $mg.L^{-1}$ and 50 $mg.L^{-1}$ of Streptomycin sulfate, Chloramphenicol, Tiabendazole, and Carbendazim) modified from Lopez-Llorca & Duncan (1986). For evaluating total root colonization by *P. chlamydosporia*, the same procedure was applied except for the surface disinfection step. Additionally, three plants inoculated with isolates Pc75, Pc123, Pc132 and Pc399 were selected to evaluate root colonization using quantitative PCR (qPCR). These isolates were selected because of their different capabilities for producing chlamydospores. DNA was extracted from surface disinfected and non-disinfected roots to assess endophytic and total colonization, respectively.

For this purpose, roots were lyophilized and then ground in liquid Nitrogen. Ground roots (200 mg) were incubated at 65°C for 1 h with CTAB Buffer (100 mM Tris-HCl pH=8.4, 1.4 M NaCl, 25 mM EDTA, 2% of polivinylpyrrolidone (PVP) and 2% of hexadecyl-trimethyl-ammonium bromide). Extracts were purified in 1 vol of phenol-chloroform-isoamyl alcohol (IAA), 25:24:1, and then in 1 volume of chloroform-IAA, 24:1. DNA was precipitated with one volume of Isopropanol. DNA pellets were washed in 70% ethanol, air hood dried and re-suspended in DNAse free water. The DNA was visualized in 1% agarose gels stained with GelRed (Biotools, UK), quantified using a nanodrop ND 1000 (Thermoscientific) and stored at 4°C until used.

In qPCR experiments, VCP1-1F (CGCTGGCTCTCTCACTAAGG) and VCP1-2R (TGCCAGTGTCAAGGACGTAG) primers (Lopez-Llorca *et al.,* 2010) were used to amplify a partial region of the VCP1 gene of *P. chlamydosporia*. Reactions were performed in 10 µL with 40 ng of root DNA, 300 nM primers and 1× SYBR Green master mix (Roche). All samples were diluted in 0.01% DEPC treated water. Reactions were performed in triplicate in a Thermal Cycling StepOnePlus (Applied Biosystem, Foster City, CA, USA) using the following temperature cycle: Initial denaturation step 95°C for 5 min, 45 cycles at 95°C for 30 s followed by 62°C during 30 s and 72°C for 30 s. The dissociation fusion curve was performed at the end of the reaction to confirm that the signal was the result of a unique amplification product. PCR products were visualized in 2% agarose gels to view the size of amplified fragment. For standard curve construction, serial dilutions were prepared from 100 ng to 10 pg of genomic DNA from *P. chlamydosporia* in 40 ng of tomato root DNA. The cycle threshold (CT) values obtained per wells containing

total DNA (root DNA) were correlated with CT values in the standard curve to calculate the quantity of fungal DNA *vs* total DNA.

Colorimetric assay for IAA determination

A colorimetric assay with some modifications was used to test IAA production (Tsavkelova *et al.,* 2007) by *P. chlamydosporia* isolates. The fungus inoculum consisted of $1x10^6$ conidia from each isolate in 20 mL of potato dextrose broth (PDB) (Beckton & Dickinson) supplemented with 2 $mg.mL^{-1}$ of L-tryptophan (Sigma) in a 50 mL sterile falcon tube. Tubes were then incubated in dark at 25°C and 170 rpm during 21 days. Mycelium was then removed from culture broth by centrifugation at 11,000 rpm for 5 min. Supernatant was mixed with 2 volumes of Salkowski reagent (50 mL of 35% $HClO_4$ + 1 mL of 0.5M $FeCl_3.6H_2O$) and then incubated in dark for 30 min. Reactions were performed in 96-well plates (Steriline) and color change was measured at 490 nm in a GENIOS TM multiwell spectrophotometer (Tecan Männedorf, Switzerland). The IAA level was estimated by correlation in IAA standard curve which was prepared from serial dilutions of IAA (Sigma) in PDB. Three replicates per each isolate were set. Additionally, to confirm the presence of IAA in Pc399 supernatants, samples were analyzed by UPLC in a spectrometer Q-Exactive Orbitrap (Thermofisher) in the Institute of Molecular and Cell Biology of Plants (IBMCP, Valencia, Spain).

Mineral phosphate solubilization

Mineral phosphate solubilization activity was assayed on solid medium supplemented with calcium phosphate (glucose 10 $g.L^{-1}$, NH_4Cl 5 $g.L^{-1}$, NaCl 1 $g.L^{-1}$, $MgSO_4.7H_2O$ 1 $g.lL^{-1}$, $Ca_3(PO_4)_2$ 170 $mg.L^{-1}$, pH 7.2) as in Goldstein (1986). Plates were inoculated in the center with a 5 mm diameter plug from the edge of a 20 day-old colony of individual *P. chlamydosporia* isolates and then incubated at 25°C, for 3 days. Phosphate solubilization activity was then expressed as (1-C/H), where (C) was colony diameter and

(H) diameter of the degradation halo (Olivares & Lopez-Llorca, 2002). Three plates per *P. chlamydosporia* isolate were scored. The experiment was carried out twice.

Statistical analysis

The plants experimental designs were completely randomized. All data were analyzed by variance analysis (ANOVA) to determine differences between treatments. Non-normal distribution data were transformed with Log and √X+1 or compared using a non-parametric test (Kruskal Wallis). Fisher´s LSD or Wilcox test were used for pair-wise comparisons. The analysis was performed using R Version 3.0.2 (R core team 2013) or IBM SPSS Statistics 20. We consider a $P < 0.05$ acceptable for statistical significance.

Results

Pochonia chlamydosporia increased the number of secondary roots of tomato seedlings

We investigated the effect of nine *P. chlamydosporia* isolates on total fresh weight (TFW) and number of secondary roots (NSR) of tomato seedlings (8 dai). The effect of *P. chlamydosporia* on growth of tomato seedlings was dependent on both the isolate and the nutrient medium (Table 2). Isolates Pc21, Pc132, Pc399 and Pc4624 significantly increased ($P<0.01$) total fresh weight (TFW) of tomato seedlings with respect to controls, Pc64, Pc75 and Pcat reduced ($P<0.05$) TFW. The rest of the isolates had not shown significant differences in Gamborg´s medium. In Gamborg´s (1/10), Pc21, Pc64, Pc69, Pc123, Pc132, Pc399 and Pc4624 significantly increased ($P<0.05$) TFW, on the contrary Pc75 decreased ($P<0.001$) TFW of tomato plants. Seedlings inoculated with isolate Pcat not showed differences with respect to control. Our results showed that in Gamborg´s medium, seedlings inoculated with Pc21, Pc123, Pc132 and Pc399 significantly increased ($P<0.05$) the number of secondary roots (NSR) with respect to control. In Gamborg´s 1/10 medium, seedlings inoculated with most *P. chlamydosporia* isolates (Pc21, Pc64, Pc123, Pc132, Pc399 and Pc4624) showed higher NSR ($P<0.05$) than controls. *P. chlamydosporia*

inoculation had no effect on tomato seed germination parameters (emergence of radicle, hypocotyl and cotyledons). This was true both for Gamborg´s and Gamborg´s 1/10 media (data not shown). Most *P. chlamydosporia* isolates were able to promote plant development. All nine isolates of *P. chlamydosporia* were fully capable to colonize the root surface of the seedlings. Figure 1 shows a tomato root 8 dai with *P. chlamydosporia* stained with Calcofluor White. The root shows abundant hyphae associated with the formation of dictyochlamydospores on the rhizoplane.

Pochonia chlamydosporia promotes root development of tomato plantlets

Pochonia chlamydosporia Pc64, Pc69, Pc123, Pc399 and Pcat significantly increased ($P<0.05$) fresh root weight (FRW) of tomato plantlets 21 dai (Table 3). On the contrary, none of the *P. chlamydosporia* isolates used in this study had any effect on maximum root length (MRL) of tomato plantlets. Regarding shoot development, except for Pc75 which reduced maximum shoot length MSL ($P<0.01$), the rest of the isolates display no effect on MSL neither on FSW or DSW.

Pochonia chlamydosporia increases shoot, root length and root weight of tomato plants in greenhouse experiment

In Experiment 3, tomato plants were harvested 90 dai. Growth parameters scored for those plants are shown in Table 4. *P. chlamydosporia* (Pc21, Pc64, Pc69, Pc123, Pc399 and Pc4624) significantly increased ($P<0.01$) FRW of tomato plants but also promoted ($P<0.05$) root length (MRL) (Pc123 and Pc4624) with respect to uninoculated plants. Regarding shoot development Pc399 inoculated plants displayed significantly larger ($P<0.05$) MSL values than controls. The rest of *P. chlamydosporia* isolates increased MSL although these values were not significantly different with respect to control plants. FSW showed no significant differences ($P<0.05$) in plants inoculated with *P. chlamydosporia* isolates.

Pochonia chlamydosporia reduces flowering and fruiting times

In experiment 3, tomato plants were left to yield (150 dai). We scored flowering and fruiting times (Fig. 2). We found that in tomato plants inoculated with *P. chlamydosporia* (Pc21, Pc69, Pc75 and Pc132) flowering time was significantly reduced (5 days sooner than in uninoculated controls on average) (Fig. 2a). We also found that fruiting time was also significantly (P<0.05) reduced in *P. chlamydosporia* (Pc64, Pc69, Pc75, Pc399, Pc4624 and Pcat). Most isolates (Pc64, Pc69, Pc75, Pc399, Pc4624 and Pcat) reduced 8d on average tomato fruiting time. However, two of these isolates (Pc64 and Pc75) further reduced fruiting time (ca. 12 days on average) (Fig. 2b).

Pochonia chlamydosporia increases root weight and mature fruit weight

In Experiment 3, tomato plants were left to yield (150 dai). *P. chlamydosporia* inoculated plants (Pc21, Pc64 and Pc75) significantly increased (P<0.05) root weight (Table 5). Regarding fruit yields, *P. chlamydosporia* isolates Pc64, Pc69, Pc75 and Pcat significantly increased (P<0.05) fruit numbers at stage 4 (previous to full maturation >45mm in diameter). *P. chlamydosporia* Pc64, Pc69, Pc75 and Pcat isolates significantly increased (P<0.05) number of marketable fruits (fully matured) with respect to uninoculated controls. Finally, Pc64, Pc69, Pc75 and Pcat significantly increased (P<0.01) mature fruit weight per plant. Other parameters also scored related with crop yield such as total number of fruits and total fruit weight per plant showed no significant differences with respect to control in plants inoculated with *P. chlamydosporia*.

Pochonia chlamydosporia isolates differ in rhizosphere competence

Total colonization of tomato roots (21 dai) by *P. chlamydosporia* determined by using culturing technique was 100% for all isolates tested. Most of *P. chlamydosporia* isolates (Pc21, Pc69, Pc75, Pc123, Pc132, Pc399 and Pcat) also displayed endophytic colonization. Pcat was the best tomato root colonizer with a 60% root endophytic colonization followed by the other isolates with values between 20–40% (Fig. 3). qPCR,

unlike culturing, revealed differences in tomato total root colonization among *P. chlamydosporia* isolates. Fig. 4 shows that for the *P. chlamydosporia* isolates tested (Pc75, Pc123, Pc132 and Pc399) there were differences in the total root colonization, isolate Pc132 was the best root colonizer followed by Pc123, Pc399 and Pc75 isolates (Fig. 4a). Endophytic root colonization which was 2–7 folds lower than total colonization also revealed differences between isolates. The best root endophytic colonizer was isolate Pc399, followed by Pc132, Pc123 and Pc75 (Fig. 4b).

Culturing was used to evaluate root colonization (90 dai) by *P. chlamydosporia,* however due to the presence of other microorganisms the fungus could not be detected (data not shown). On the contrary, using qPCR, *P. chlamydosporia* was detected at very low amounts (8–20 fold less) for total root colonization in 90 dai with respect to 21 dai plant root. Plants inoculated with Pc132 showed the largest colonization with 2-fold more pg of fungal DNA than plants inoculated with Pc75. Moreover, Pc123 and Pc399 had similar values of fungus colonization estimated by qPCR (Figure 4c). Regarding endophytic colonization was 1.5–2 fold less than total colonization; the largest endophytic colonization was again obtained with Pc399, followed by Pc132, Pc123 and Pc75 (Fig. 4d).

IAA production and phosphate solubilization by *P. chlamydosporia*

Pochonia chlamydosporia was able to synthetize IAA in liquid culture supplemented with Tryptophan (Table 6). There were differences in IAA production among *P. chlamydosporia* isolates. The maximum IAA production was displayed by Pcat (267.62 $\mu g.mL^{-1}$) followed by Pc4624, Pc69 and Pc21 (252.23, 237.62 and 228.07 $\mu g.mL^{-1}$, respectively). IAA production was also verified by UPLC for Pc399 (264 $\mu g.mg^{-1}$ mycelium). All *P. chlamydosporia* isolates tested were able to rock phosphate solubilize even at very early stages of growth (Table 6). As for IAA, Pc4624 and Pcat were the best phosphate solubilizers (0.72, 0.65 arbitrary index) strains.

Discussion

Pochonia chlamydosporia is a cosmopolitan fungus (Gams & Zare, 2001). Its capacity to produce extracellular enzymes, parasitize nematode eggs and saprotrophic growth varies largely among isolates (Bourne *et al.,* 1996; Kerry, 2000; Olivares-Bernabeu & Lopez-Llorca, 2002; Morton *et al.,* 2003; Esteves *et al.,* 2009). The fungus also colonizes plant roots as a true endophyte (Bordallo *et al.,* 2002) of both monocotyledon (Maciá-Vicente *et al.,* 2008; Lopez-Llorca *et al.,* 2010) and dicotyledon plant roots (Bordallo *et al.,* 2002; Manzanilla-Lopez *et al.,* 2011; Escudero & Lopez-Llorca, 2012). Most studies related with the effect of *P. chlamydosporia* on crop yield have been performed in plant parasitic nematodes (PPN) infested plants (Rao *et al.,* 2004; Khan *et al.,* 2005; Wang *et al.,* 2005; Singh *et al.,* 2011; Muthulakshmi *et al.,* 2012; Chaya & Rao, 2012). Most of the above mentioned studies on the effect of *P. chlamydosporia* on crops were carried out with a single isolate. On the contrary, in our work, we have chosen a selection of *P. chlamydosporia* isolates from worldwide origin and we have investigated their effects on tomato growth promotion, development and yield.

In our study, *P. chlamydosporia* increased number of secondary roots of tomato seedlings. Similar results were found for other biocontrol agents with endophytic capabilities such as *Trichoderma virens* (Contreras-Cornejo *et al.,* 2009) and the endophyte *Piriformospora indica* (Druege *et al.,* 2007) in *Arabidopsis thaliana* and ornamental plants, respectively.

Using culturing methods, Maciá-Vicente *et al.,* (2008) found that single inoculative application of *P. chlamydosporia* in young barley plants resulted in a reduction of the presence in the rhizosphere over time. This effect was also shown in tomato with a GFP-tagged *P. chlamydosporia* strain (Escudero & Lopez-Llorca, 2012). Multiple applications of *P. chlamydosporia* reduced the nematode gall rating and the egg population in tomato roots (Sorribas *et al.,* 2003). For this reason, in this work we have combined an inoculative strategy using hyphae in tomato plantlets together with two inundative soil inoculations with chlamydospores in potted plants in the greenhouse. Following previous works, we used the amount of *P. chlamydosporia* inoculum found in suppressive soils for PPNs (Kerry & Bourne, 2002).

This strategy resulted in a sustained tomato root growth promotion. Pc21 isolate was found to promote root growth from seedlings to fully matured tomato plants. Most isolates were found to increased fresh root weight in at least two of the four tomato stages of growth consider (seedlings, plantlets, plants and fruiting plants). We have finally evaluated the effect of *P. chlamydosporia* isolates on flowering and fruiting times and yield of a commercial tomato cultivar. Most *P. chlamydosporia* isolates significantly reduced flowering and fruiting times. Flowering time reduction by AM in tomato plants (Micro-tom) has also been reported (Salvioli *et al.,* 2012). In our study, some *P. chlamydosporia* isolates reduced ca. 18% the fruiting time of uninoculated tomato plants. In future studies, the agronomic significance of these results should be fully evaluated. In our study, *P. chlamydosporia* did not improve yield, similar to previous studies in double-cropping lettuce-tomato plants (Verdejo-Lucas *et al.*, 2003), as well as in Sorribas *et al*. (2003), were multiple applications of *P. chlamydosporia* reduced nematodes gall rating and egg population but the tomato yield not showed significantly differences when compared with untreated plants. However, studies realized by Khan *et al*. (2012) showed than *P. chlamydosporia* increased the yield of eggplants with the addition of dry neem leaves or lettuce experiment realized by Dias-Arieira *et al*. (2011) were the vegetative parameters of the plant was increased in areas of lower soil fertility. These results are similar to ours were the number and weight of mature fruits was higher in plants inoculated with *P .chlamydosporia.* Isolates Pc21, Pc399, Pc75 and Pcat, promoted root development, increasing number of secondary roots and FRW in greenhouse conditions, and all of them had the capabilities to produce IAA and solubilize phosphate. These features are adequate necessary to enhance nematode tolerance, promote growth and finally increase the yield of tomato plants. Future work should be carried out on their compatibility for attempts their use in combination.

The mechanism by which *P. chlamydosporia* promotes tomato growth and development is currently unknown. However, all isolates of *P. chlamydosporia* primed *in vitro* with tryptophan as precursor have been shown to produce indole acetic acid. This capacity has been reported from endophytic bacteria (Verma *et al.,* 2001; Tsavkelova *et al.,* 2007; Hashtroudi *et al.,* 2013) and fungi (Salas-Marina *et al.,* 2011; Radhakrishnan *et al.,*

2013), including mycorrhizae (Mohandas *et al.,* 2013). Future studies should investigate the involvement of plant hormones in the plant growth promotion effect of *P. chlamydosporia.*

Phosphorus is a key element for plants since it is the least available of all essential nutrients in soil (Fusconi, 2014). In our study, all *P. chlamydosporia* isolates tested were able to solubilize mineral phosphate. This activity is commonly found in plant growth promoting microorganisms (Vassilev *et al.,* 2006) and could, at least partially, account for the ability of *P. chlamydosporia* to increase plant growth.

Our results support that *P. chlamydosporia* is a nematophagous fungus with plant growth promoting capabilities of potential agronomical value. Several aspects such as inoculation approaches, increase of rhizosphere competence and soil receptivity as well as the cellular, physiological and molecular basis of root growth promotion and yield increase should be fully investigated. This will enhance the role of *P. chlamydosporia* as microbial inoculant in sustainable agriculture. *P. chlamydosporia* growth promotion in crops is particularly relevant in a scenario of global climate change.

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Tables

Table 1 *P. chlamydosporia* isolates used in this study

Isolate ref.	Host Origin	Location
Pc21	*Meloidogyne* spp. eggs	Italy
Pc64	*Meloidogyne* spp. eggs	Spain
Pc69	*Heterodera evenae* eggs	New Zealand
Pc75	*Heterodera schachtii* eggs	Spain
Pc123	*Heterodera evenae* eggs	Spain
Pc132	*Meloidogyne* spp. eggs	Kenya
Pc399	*Meloidogyne* spp. eggs	China
Pc4624	Soil organic matter	Australia
Pcat	*Meloidogyne* spp. eggs	Cuba

Table 2 Effect of *P. chlamydosporia* isolates (Pc21, Pc64, Pc69, Pc75, Pc123, Pc132, Pc399, Pc4624 and Pcat) from worldwide origin (see Table 1) on the development of tomato seedlings (8 dai) inoculated with the fungus.

Treatment	Gamborg´s		Gamborg´s 1/10	
	TFW (mg)	**NSR**	**TFW (mg)**	**NSR**
Control	34.10 ± 1.7	3.0 ± 0.5	30.09 ± 1.4	1.7 ± 0.3
Pc21	43.91 ± 2.3**	4.5 ± 0.5**	34.12 ± 1.4*	2.3 ± 0.5**
Pc64	27.60 ± 1.4*	2.1 ± 0.3	38.83 ± 2.2***	2.4 ± 0.5***
Pc69	39.64 ± 2.4	3.8 ± 0.4	35.24 ± 1.4*	1.8 ± 0.4
Pc75	21.32 ± 1.5***	1.8 ± 0.2*	23.00 ± 1.0***	1.7 ± 0.3
Pc123	35.87 ± 2.3	3.9 ± 0.4*	36.72 ± 2.4**	2.3 ± 0.5***
Pc132	44.30 ± 1.8**	6.2 ± 0.5***	37.30 ± 1.5**	2.4 ± 0.5***
Pc399	43.51 ± 2.5**	4.3 ± 0.4**	37.92 ± 2.8**	2.3 ± 0.5**
Pc4624	41.52 ± 1.3*	3.4 ± 0.3	35.61 ± 1.8*	2.3 ± 0.5**
Pcat	27.04 ± 1.4**	1.5 ± 0.3**	26.80 ± 1.2	2.1 ± 0.4

Abbreviations: TFW: Total fresh weight; NSR: Number of secondary roots; Gamborg´s and Gamborg´s 1/10: Agar media supplemented with salt solution Gamborg´s B5. Bars indicate mean ± SEs of n=30 seedlings.Significant effect of inoculation at: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$ with ANOVA.dai = days after inoculation.

Table 3 Effect of *P. chlamydosporia* isolates (Pc21, Pc64, Pc69, Pc75, Pc123, Pc132, Pc399, Pc4624 and Pcat) from worldwide origin (see Table 1) on the development of tomato plantlets (21 dai) inoculated with the fungus.

Treatment	FRW (g)	MRL (g)	FSW (g)	DSW (g)	MSL (cm)
Control	1.38 ± 0.15	9.85 ± 0.43*	1.89 ± 0.13	0.18 ± 0.01	8.61 ± 0.62
Pc21	1.78 ± 0.16	8.83 ± 0.26	2.20 ± 0.18	0.22 ± 0.02	8.94 ± 0.62
Pc64	1.92 ± 0.15*	8.76 ± 0.64	2.21 ± 0.11	0.22 ± 0.02	9.51 ± 0.71
Pc69	2.22 ± 0.12**	8.81 ± 0.31	2.14 ± 0.13	0.20 ± 0.01	8.93 ± 0.4
Pc75	1.69 ± 0.12	8.91 ± 0.64	1.92 ± 0.18	0.18 ± 0.02	7.95 ± 0.75**
Pc123	2.39 ± 0.24***	8.76 ± 0.62	2.16 ± 0.12	0.22 ± 0.01	8.86 ± 0.23
Pc132	1.73 ± 0.11	8.89 ± 0.35	2.00 ± 0.17	0.19 ± 0.02	8.33 ± 0.99
Pc399	1.95 ± 0.16*	9.01 ± 0.22	2.06 ± 0.14	0.19 ± 0.02	8.88 ± 0.29
Pc4624	1.93 ± 0.12*	8.85 ± 0.36	2.07 ± 0.16	0.19 ± 0.02	8.68 ± 0.88
Pcat	1.97 ± 0.16*	8.90 ± 0.22	2.12 ± 0.16	0.20 ± 0.01	8.63 ± 0.63

Abbreviations: FRW: fresh root weight; MRL: maximum root length; FSW: fresh shoot weight; DSW: dry shoot weight and MSL: maximum shoot length. The data are based on ~~3~~ three independent experiments with 10 seedlings each. Bars indicate mean ± SE of n=30 seedlings. Significant effect of inoculation at: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. dai = days after inoculation.

Table 4 Effect of *P. chlamydosporia* isolates (Pc21, Pc64, Pc69, Pc75, Pc123, Pc132, Pc399, Pc4624 and Pcat) from worldwide origin (see Table 1) on the development of tomato plants (90 dai) inoculated with the fungus.

Treatment	FRW (g)	MRL (cm)	FSW (g)	MSL (cm)
Control	153 ± 9.7	26 ± 0.7	360 ± 15.50	57. ± 1.4
Pc21	255 ± 22.7***	26 ± 0.3	370 ± 21.73	63 ± 1.1
Pc64	220 ± 12.5***	28 ± 0.7	309 ± 16.25	62 ± 1.3
Pc69	189 ± 11.5*	26 ± 0.5	362 ± 18.46	62 ± 1.3
Pc75	169 ± 9.5	28 ± 0.6	362 ± 25.46	62 ± 1.2
Pc123	241 ± 52.5***	29 ± 0.5*	361 ± 16.23	64 ± 1.8
Pc132	148 ± 8.52	28 ± 0.6	311 ± 12.14	62 ± 2.2
Pc399	252 ± 23.9***	28 ± 0.7	329 ± 12.66	65 ± 1.8*
Pc4624	205 ± 14.3**	29 ± 0.6*	373 ± 16.42	62 ± 1.7
Pcat	174 ± 10.8	28 ± 0.6	374 ± 24.24	62 ± 1.4

Abbreviations: MSL: maximum shoot length. MRL: maximum root length. FSW: fresh shoot weight. FRW: fresh root weight. Bars indicate mean ± SE of n=30 seedlings. Significant effect of inoculation at: *$P < 0.05$, **$P < 0.01$ and ***$P < 0.001$. dai = days after inoculation.

Table 5 Effect of *P. chlamydosporia* isolates (Pc21, Pc64, Pc69, Pc75, Pc123, Pc132, Pc399, Pc4624 and Pcat) from worldwide origin (see Table 1) on the development and yield of tomato plants (150 dai) inoculated with the fungus.

Treatment	FRW	FSW	E4	MF	NFP	TFWP	MFWP
Control	513 ± 32	661 ± 29	4.2 ± 2.3	3.9 ± 2.7	15 ± 6.8	370 ± 98	164 ± 109
Pc21	693 ± 47*	693 ± 36	6.5 ± 3.2	3.8 ± 2.2	17 ± 6.7	392 ± 141	169 ± 123
Pc64	641 ± 34*	613 ± 42	10.5 ± 3.9*	8.3 ± 4.2*	18 ± 7.0	489 ± 163	306 ± 114**
Pc69	577 ± 24	657 ± 19	10 ± 3.6*	7.0 ± 2.4*	24 ± 9.9	490 ± 157	257 ± 120**
Pc75	667 ± 35*	707 ± 24	9.3 ± 4.6*	6.4 ± 4.1*	20 ± 4.3	474 ± 160	266 ± 165*
Pc123	574 ± 65	705 ± 29	5.3 ± 2.4	2.8 ± 1.3	18 ± 7.2	330 ± 134	145 ± 54
Pc132	534 ± 42	690 ± 21	6.5 ± 2.0	4.5 ± 2.2	17 ± 6.0	399 ± 94	200 ± 99
Pc399	447 ± 27	674 ± 38	7.3 ± 3.3	5.2 ± 3.1	19 ± 7.5	435 ± 168	243 ± 145
Pc4624	520 ± 40	694 ± 20	7.9 ± 3.2	5.2 ± 2.2	20 ± 8.9	452 ± 141	210 ± 106
Pcat	503 ± 27	640 ± 28	9.4 ± 3.8*	6.3 ± 4.0*	18 ± 5.3	453 ± 141	260 ± 163*

Abbreviations: FRW: fresh root weight; FSW: fresh shoot weight; E4: Stage 4 (number of fruits with diameter >45mm); MF: Stage 5 (number of marketable fruits); NFP: Number of fruits per plant; TFWP: total fruit weight per plant; MFWP: Mature fruit weight per plant. The data are based on ~~3~~ three independent experiments with 10 seedlings each. Bars indicate mean ± SE of n=30 seedlings. Significant effect of inoculation at: **P* < 0.05 and ***P* <0.01. dai = days after inoculation

Table 6 Indole Acetic Acid production and mineral phosphate solubilization by *P. chlamydosporia* isolates (Pc21, Pc64, Pc69, Pc75, Pc123, Pc132, Pc399, Pc4624 and Pcat) from worldwide origin (see Table 1)

***P. chlamydosporia* isolate**	**IAA (µg.ml^{-1})**	**Phosphate solubilization index***
Pc21	228.07 ± 16.02^{bc}	0.41 ± 0.06^{ab}
Pc64	158.95 ± 37.37^{a}	0.33 ± 0.14^{a}
Pc69	237.62 ± 9.24^{bd}	0.50 ± 0.00^{b}
Pc75	163.21 ± 31.21^{a}	0.65 ± 0.05^{cd}
Pc123	215.93 ± 6.91^{b}	0.52 ± 0.04^{b}
Pc132	210.50 ± 11.70^{b}	0.58 ± 0.05^{bc}
Pc399	205.73 ± 19.97^{b}	0.57 ± 0.03^{bc}
Pc4624	252.23 ± 21.96^{de}	0.72 ± 0.08^{d}
Pcat	267.62 ± 8.89^{e}	0.65 ± 0.05^{cd}

(*) Phosphate solubilization index is expressed as (1-C/H) being (C) colony diameter and (H) the diameter of the halo caused by phosphate solubilization. Numbers followed with different letters in each column are significantly different ($P< 0.05$).

Figure legends

Figure 1 Root fragment of a 8 day-old tomato seedling inoculated with *P. chlamydosporia* (Pc21). Arrows point hyphae and chlamydospores on the root surface. Insert: General view of tomato seedlings on Gamborg´s medium with a colony of Pc21. Bar = 50 µm Abbreviation: dai = days after inoculation.

Figure 2 Effect of *P. chlamydosporia* isolates (Pc21, Pc64, Pc69, Pc75, Pc123, PC132, Pc399, Pc4624 and Pcat) on flowering (Fig. 4a) and fruiting times (Fig. 4b) of tomato plants (150 dai). The data are based on 3 independent experiments with 10 seedlings each. Bars indicate mean ± SE of n=30 seedlings. Abbreviation: dai = days after inoculation. Asterisks above bars indicate significant differences ($P<0.05$).

Figure 3 Endophytic root colonization of tomato plantlets (21dai) by *P. chlamydosporia* (Pc21, Pc64, Pc69, Pc75, Pc123, Pc132, Pc399, Pc4624 and Pcat) isolates determined by culturing technique. Each value is the mean ± SE of n=3 roots. Different letters in the columns represent differences between treatments ($P<0.05$). Abbreviation: dai = days after inoculation.

Figure 4 Detection of *P. chlamydosporia* (Pc75, Pc123, Pc132 and Pc399) DNA in tomato roots using real-time quantitative PCR. Total and endophytic colonization were measured from non-sterile and surface disinfected roots respectively; a) The figure shows pg of *P. chlamydosporia* DNA in non-disinfected tomato roots of 21 dai, b) Shows the endophytic colonization (pg of *P. chlamydosporia* DNA in surface disinfected tomato roots of 21 dai), c) Shows total root colonization of 90 dai plants (pg of *P. chlamydosporia* DNA in non-disinfected tomato roots), d) endophytic root colonization of 90 dai plants (pg of *P. chlamydosporia* DNA in surface disinfected tomato roots). Each value (±SE) represents the mean of three roots inoculated with each isolate. Different letters in the columns represent differences between treatments ($P<0.05$). Abbreviation: dai = days after inoculation.

Figure 1

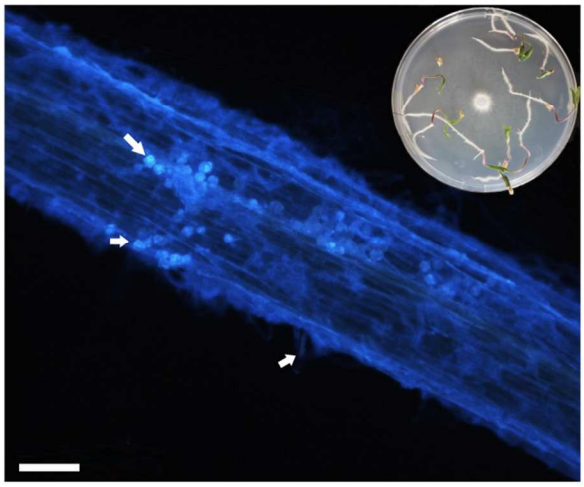


Figure 2

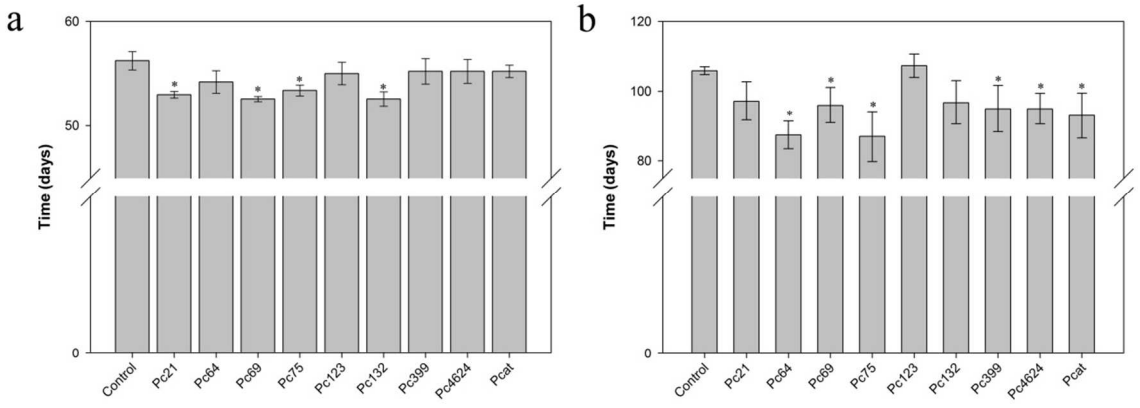


Figure 3

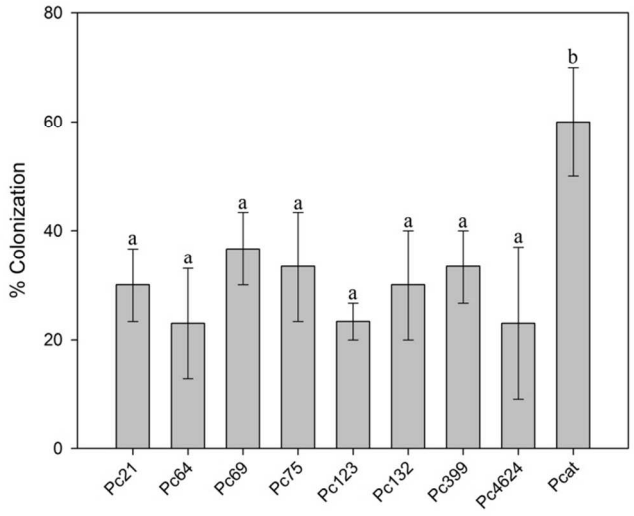
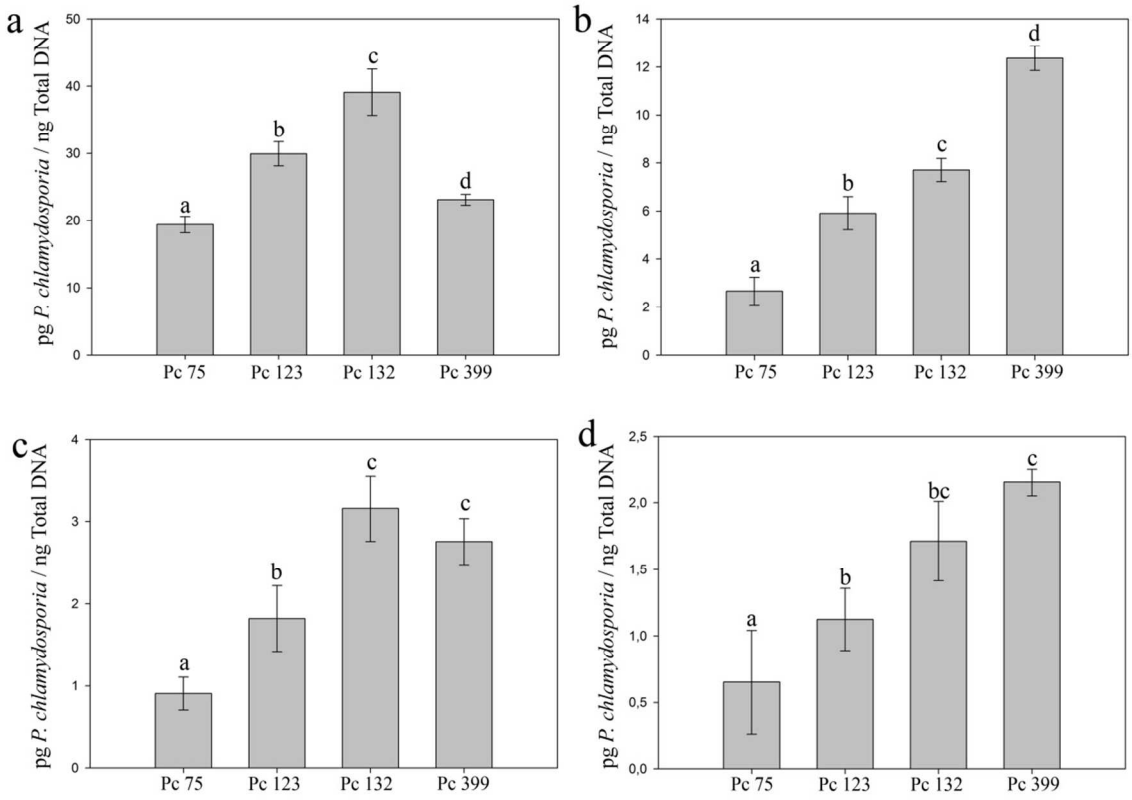
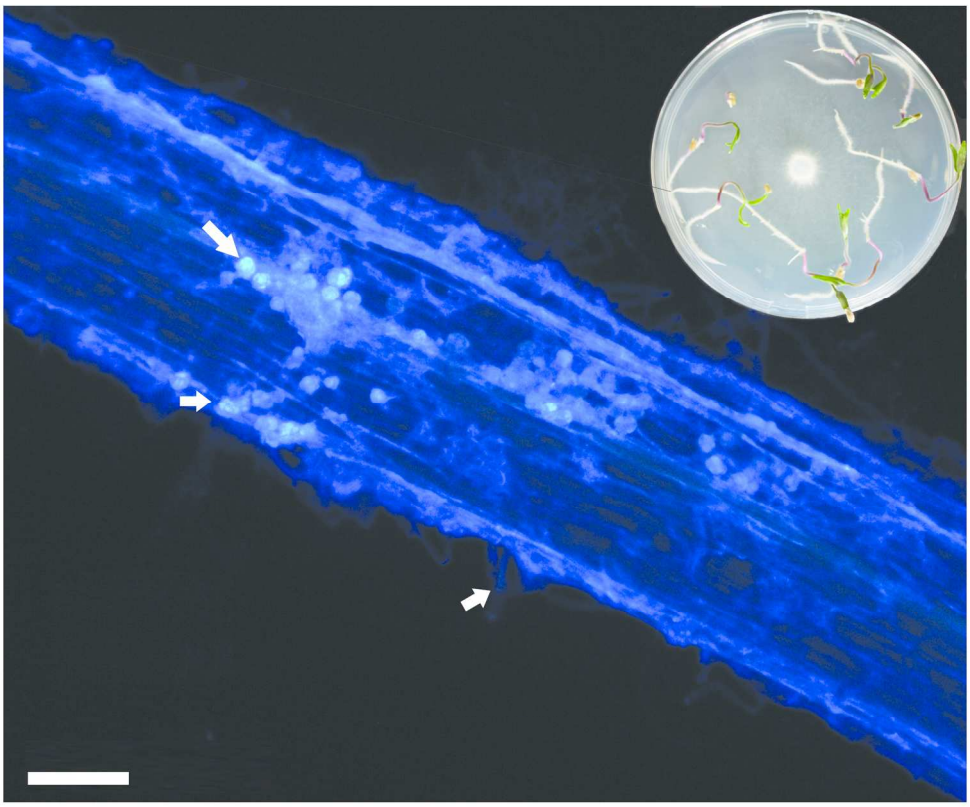


Figure 4





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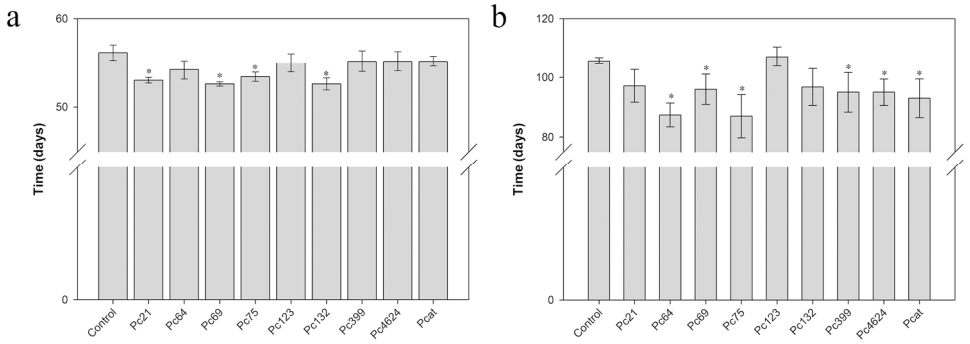

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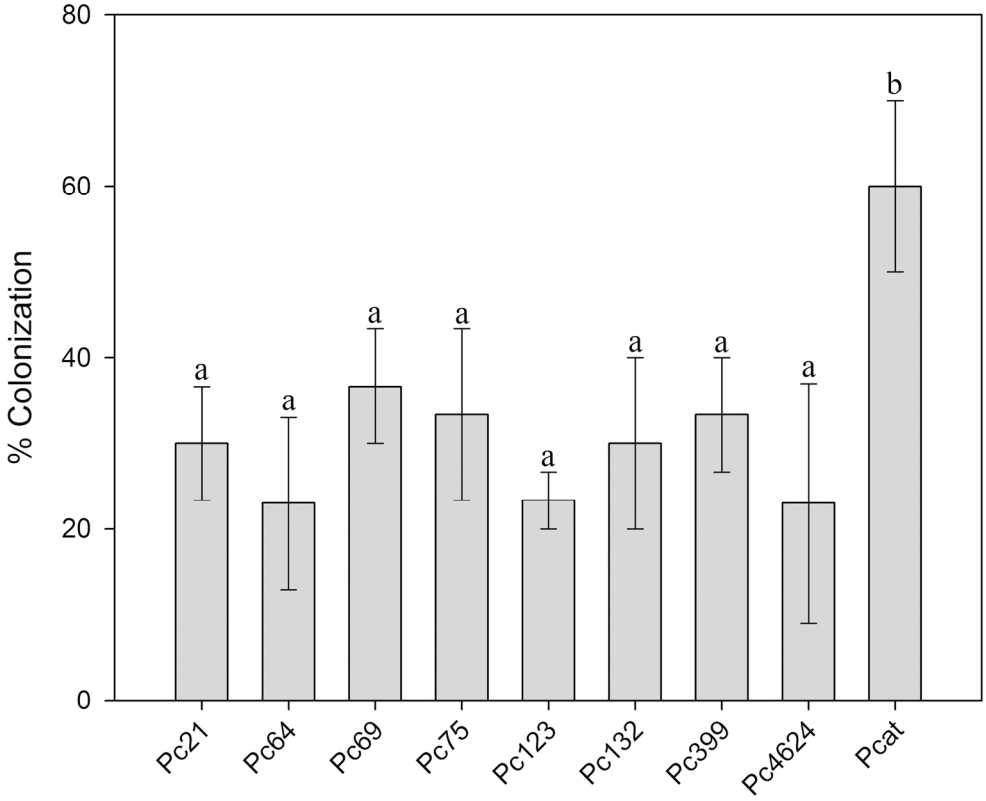

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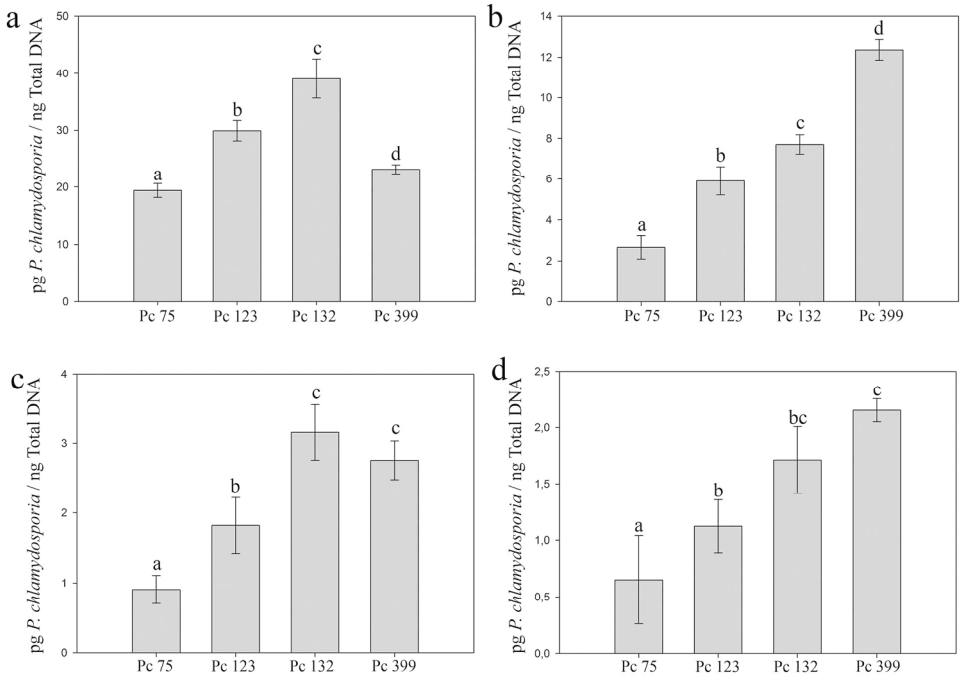

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